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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 47/48 — A2

(11) International Publication Number: WO 97/36616

(43) International Publication Date: 9 October 1997 (09.10.97)

(21) International Application Number: PCT/GB97/00917

(22) International Filing Date: 1 April 1997 (01.04.97)

(30) Priority Data:
9606975.2 — 2 April 1996 (02.04.96) — GB

(71) Applicant (*for all designated States except US*): THE UNIVERSITY OF BIRMINGHAM [GB/GB]; Edgbaston, Birmingham B15 2TT (GB).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): SEYMOUR, Leonard, William [GB/GB]; 59 Max Road, Quinton, Birmingham B32 1LD (GB). SCHACHT, Etienne, Honore [BE/BE]; Rijsseveldstraat 99, B-8140 Staden (BE). SOYEZ, Heidi [BE/BE]; Leeuweriken Street 221, B-8400 Oosterd (BE).

(74) Agents: PEARCE, Anthony, Richmond et al.; Marks & Clerk, Alpha Tower, Suffolk Street Queensway, Birmingham B1 1TT (GB).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: ANTI-TUMOUR AGENT

(57) Abstract

An anti-tumour agent comprises a macromolecular carrier moiety, an active alkylating moiety which is a nitrogen mustard and a stabilising moiety which links the active alkylating moiety with the carrier moiety. The stabilising moiety is an oligopeptide which is capable of being cleaved by a tumour-associated protease and which stabilises the active alkylating moiety by electron withdrawal and/or by inducing formation of aggregates.

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ANTI-TUMOR AGENT

This invention relates to an anti-tumor agent and is more particularly concerned with an anti-tumor agent utilising an active alkylating moiety selected from the class of compounds referred to as "nitrogen mustards", for example N,N-di(2-chloroethyl)-4-phenylenediamine (hereinafter sometimes referred to as "PDM") and active analogues thereof. Nitrogen mustards are so called because of their ability to form cyclic onium salts in an analogous manner to the ability of mustard gas to form cyclic sulphonium salts. Nitrogen mustards have not previously found acceptability for use as anti-tumor agents because of their high systemic toxicity and short half-life.

It is an object of the present invention to provide an improved anti-tumor agent.

According to the present invention, there is provided an anti-tumor agent comprising a macromolecular carrier moiety; an active alkylating moiety which is a nitrogen mustard; and a stabilising moiety linking the active alkylating moiety with the carrier moiety, said stabilising moiety being an oligopeptide which is capable of being cleaved by a tumour-associated protease and which stabilises the active alkylating moiety by electron withdrawal and/or by inducing formation of aggregates.

By the use of the stabilising moiety and the carrier moiety, the active alkylating moiety is stabilised so that, instead of having a free-form half-life of typically about 11 minutes, when linked to the stabilising and carrier moieties as in the present invention, it has a typical half-life of about 11 hours. However, after the stabilising moiety has been cleaved

in use by the tumour-associated protease, the released alkylating moiety is very effective in killing the targeted tumor cells whilst degrading rapidly to reduce systemic toxicity.

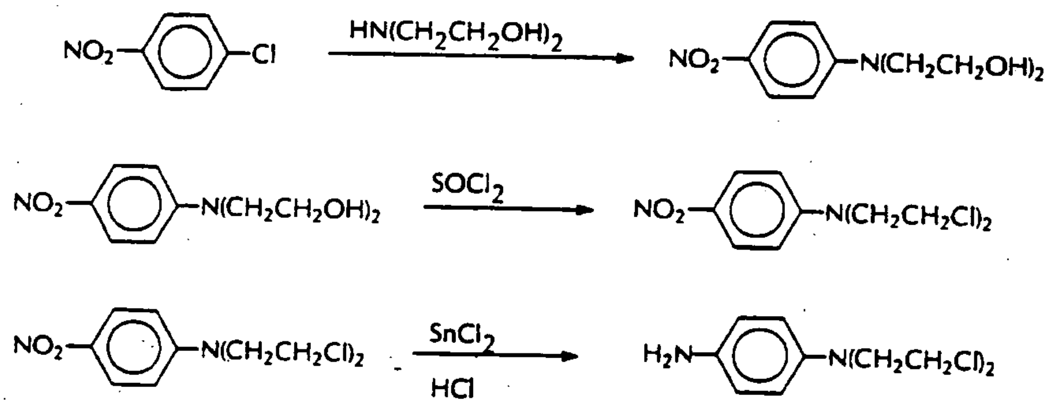
The oligopeptide is preferably one which can be cleaved by a metalloproteinase, especially gelatinase, or urokinase, for various invasive and metastatic tumours, and lysosomal enzymes for all tumours. The oligopeptide is preferably a tetra-, penta- or hexa-peptide, more preferably a tetra- or penta-peptide, and most preferably a tetra-peptide. Hereinafter, the peptides in the oligopeptide will be identified by reference to their positions relative to the active alkylating moiety, with the peptide nearest the active alkylating moiety being designated as peptide 1, the adjacent peptide being designated as peptide 2, and so on. Peptide 1 is preferably Leu, although Gly may be possible. Peptide 2 is preferably Ala, particularly when peptide 1 is Leu, although Leu may be possible if peptide 1 is Gly. Peptide 3 is preferably Phe or Leu, particularly when peptide 1 is Leu and peptide 2 is Ala; and most preferably petide 3 is Phe, particularly when peptide 1 is Leu and peptide 2 is Ala. Peptide 4 is preferably Gly or Ala and most preferably is Gly.

Currently, the most promising oligopeptide terminates with, or consists of, the tetra-peptide, Gly-Phe-Ala-Leu, although it is considered that oligopeptides terminating with, or consisting of, the tetra-peptide Gly-Phe-Leu-Gly or Ala-Leu-Ala-Leu may be used.

The macromolecular carrier moiety is preferably hydrophilic and is most preferably a polymeric moiety such as poly-[N^5-(2-hydroxyethyl)-L-glutamine] (PHEG) or any other per se known macromolecular moiety,

for example as described in EP-A-0187547. Other carriers, eg dextran, may be used.

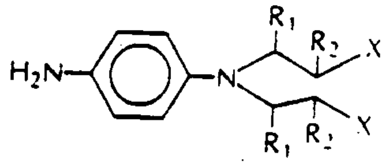
The active alkylating moiety is a nitrogen mustard, for example N,N-di(2-chloroethyl)-4-phenylenediamine (PDM) which may be produced using the following reaction scheme:-



(PDM)

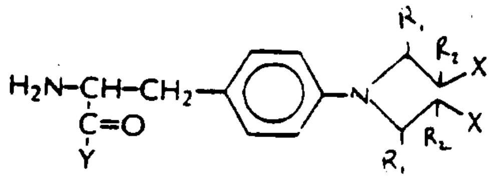
The PDM may be replaced by any active analogous mustard, for example:-

$CH_3N(CH_2CH_2Cl)_2$,



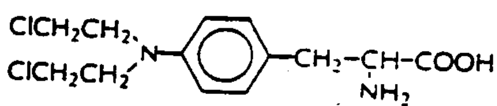
where R_1 and R_2 are independently selected from H, CH_3 or allyl and X is Cl, Br, I, mesyl or tosyl

or

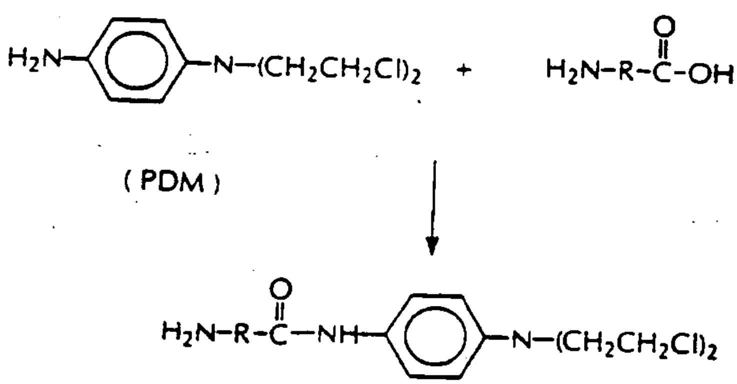


where Y is OH, alkoxy or NH_2, and R_1, R_2 and X are as defined above

An example of a nitrogen mustard of the last-mentioned formula is:-



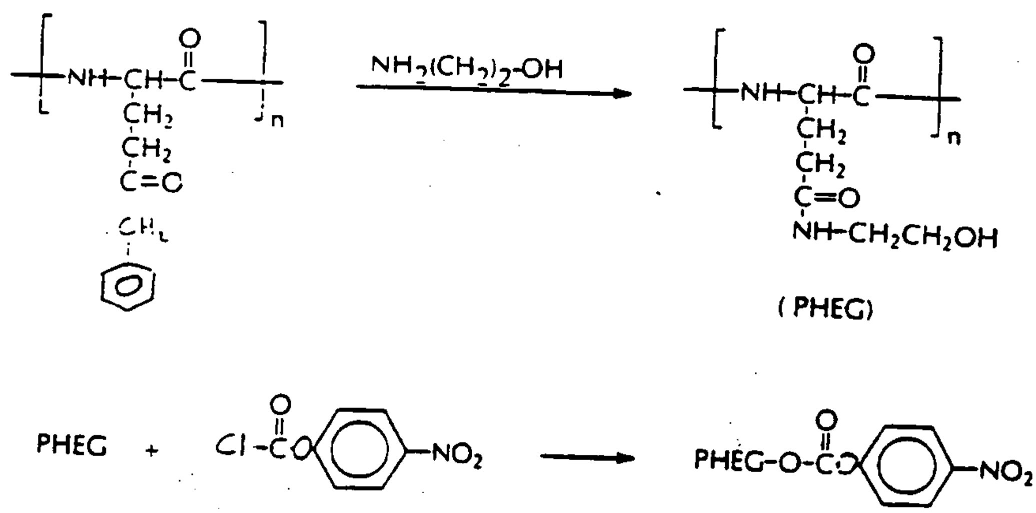
The anti-tumor agent of the present invention can be produced by first coupling the nitrogen mustard with an oligopeptide in accordance with the reaction scheme below (given, by way of example, for PDM):-



(PDM)

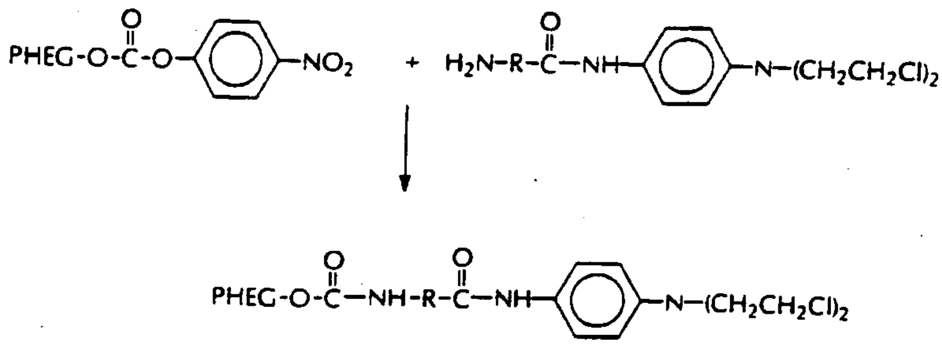
In the above scheme, R is an oligopeptide moiety as described above.

The resultant low molecular weight peptidyl-PDM derivative may then be coupled to a 4-nitrophenyl chloroformate activated poly-[N^5-(2-hydroxyethyl)-L-glutamine] (PHEG) which can be produced in accordance with the reaction scheme below:-



Other poly-L-glutamic acid derivatives can be produced in a similar way using other aminoalkanols, eg aminopropanol, in place of the aminoethanol used above.

The coupling between the peptidyl-PDM derivative and the 4-nitrophenyl chloroformate activated poly-[N^5-(2-hydroxyethyl)-L-glutamine] may be effected in accordance with the reaction scheme below:-



The anti-tumor agent may be administered intravenously, intra-peritoneally or intra-arterially.

The present invention will now be described in further detail in the following Examples.

Preparation Example

Poly-[N^5-(2-hydroxyethyl)-L-glutamine] (PHEG) was used in this Example as the macromolecular carrier because it is non toxic, non immunogenic and possesses primary hydroxyl side groups which can be easily converted into reactive carbonate esters by reaction with a chloroformate-type compound (eg 4-nitrophenyl chloroformate). Furthermore, it has been demonstrated that this synthetic polyaminoacid is a good substrate for lysosomal enzymes, preventing unwanted accumulation of the polymer in the body.

In a first stage, glycine was used because this yields a water-soluble, low molecular weight Gly-PDM derivative. However, Gly-Leu-Phe-PDM and Gly-Phe-Ala-leu PDM were also prepared.

Subsequently, the amine-containing peptide-PDM moieties were coupled to the chloroformate activated carrier. The hydrolytic stability of PDM, water-soluble Gly-PDM and the oligopeptide-PDM derivatives was investigated in buffers of lysosomal and physiological pH. Dynamic laser light scattering provided insight in the obtained results. Furthermore, the degradation in presence of tritosomes, cathepsin A and collagenase type IV was studied.

Materials and Methods

Chemicals

The aminoacids and peptides were obtained from Bachem Chem Co. (Bubendorf Switzerland), 4-nitrophenyl chloroformate was obtained from Merck (Darmstadt, Germany). Collagenase type IV was obtained from Sigma Chem. Co. (St. Louis, Missouri, U.S.A). All other chemicals were purchased from Acros (Beerse, Belgium).

^{1}H-NMR spectra were recorded on a Brucker 360 or 500 MHz apparatus. U.V. spectrophotometric measurements were carried out using an U.V. - Uvikon 810 apparatus (Kontron Instruments). Infrared spectra were recorded on a Perkin Elmer 1600 FTIR Series device. Determination of chloride content was done with an ORION 96-17B chloride electrode. Light scattering measurements were carried out using a DLS-700 dynamic light scattering spectrophotometer from Polymer Laboratories.

Synthesis of PDM

a) Preparation of N,N-di-(2-hydroxyethyl)-4-nitroaniline

A mixture of 23.6 g 4-nitrochlorobenzene and 29ml diethanolamine was heated at 120 °C for 6 hours. The hot mixture was poured into 800 ml of boiling water and stirred for 10 min while maintaining the temperature near boiling. The solution was cooled overnight and the solid product was collected by filtration and recrystallised from toluene/methanol to give yellow needles (9 g). The product was characterised by ^{1}H-NMR spectroscopy (360 MHz) in MeOD-d4 · $\delta = 8.05$ ppm and $\delta = 6.80$ ppm, $J = 9.41$ Hz : aromatic protons $\delta = 3.76$ ppm and $\delta = 3.66$ ppm, $J = 5.79$ Hz : CH_2 hydroxyethyl

b) Preparation of N,N-di--(2-chloroethyl)-4-nitroaniline

A mixture of 1g N,N-di-(2-hydroxyethyl)-4-nitroaniline, 350 μl pyridine, 10 ml dichloro-methane and 1.5 ml thionylchloride was refluxed for 1 hour. 10 ml water was added to the cooled solution and the organic layer was separated, washed twice with 10 ml, dried over sodium sulphate and evaporated in vacuo. The residue was crystallised from methanol to give yellow/orange needles (0.94 g). The product was characterised by ^{1}H-NMR spectroscopy in $CDCl_3$: $\delta = 8.15$ ppm and $\delta = 6.70$ ppm, $J = 9.37$ Hz : aromatic protons, $\delta = 3.90$ and $\delta = 3.70$ ppm, $J = 6.81$ Hz : CH_2 chloroethyl.

c) Preparation of N,N-di-(2-chloroethyl)-4-phenyldiamine

A mixture of 1.5g N,N-di-(2-chloroethyl)-4-nitroaniline, 4.29 g $SnCl_2$ and 30 ml concentrated HCl was refluxed for 60 min. The cooled mixture was filtered and the crystalline filter was dried at the pump before dissolving it in 150 ml water. The pH was adjusted to 8 by addition of 1 M NaOH solution and the mixture was extracted twice with 50 ml

ether which had been dried over sodium sulphate. An 0.5 M HCl solution in dry ether was dropwise added to the stirred ether solution and the precipitate was filtered and vacuum dried (1 g). The product was characterised by ^{1}H-NMR spectroscopy in D_2O : $\delta = 7.30$ ppm and $\delta = 7.05$ ppm : aromatic protons, $\delta = 3.90$ ppm and $\delta = 3.70$ ppm : CH_2 chloroethyl.

After alkaline hydrolysis, the chloride content was determined using a chloride ion selective electrode indicating the presence of the monochloro salt form of PDM.

Synthesis of PHEG

N-carboxyanhydride (NCA) was prepared by reaction of γ-benzyl-L-glutamine with diphosgene in ethylacetate. The NCA was crystallised twice from ethyl acetate/hexane. Poly-(γ-benzyl-L-glutamine) (PBLG) was prepared by polymerisation of the respective NCA in ethylacetate/dichloromethane, initiated by tri-n-butyl amine (monomer/initiator ratio 1/1) at room temperature.

After polymerisation PBLG was precipitated in excess methanol/ether (2/1 v/v), filtered and dried. Aminolysis with 2-aminomethanol in presence of 2-hydroxypyridine as a catalyst (ratio catalyst/ester groups 5/1) was carried out in DMF. PHEG was precipitated in excess ether/ethanol (4/1 v/v), filtered and dialyzed against water for 2 days. After freeze-drying, pure PHEG was obtained as a white powder. The molecular weight was determined by an HP-SEC method : columns : Tessek Hema Bio 1000, 300, 100 and 40; eluent : citrate buffer pH = 6.0 0.1 M using dextran standards (Mw = 123600, 43500, 21400 and 9890). The number and

weight average molecular weights were $M_w = 20700$ and $M_n = 14300$, respectively.

Preparation of peptide-PDM-conjugates

a) Protection of the peptide

The amino function of the aminopeptide was blocked with a t-butyloxycarbonyl group (Boc) using standard procedures, eg by the method of M. Bodanski and A. Bodanski, "The Practice of Peptide Synthesis" Springer Verlag,(1984).

b) Coupling reaction with PDM

To a solution of protected peptide (2.15 mmol) in 9 ml dry THF were added 0.24 ml of N-methylmorpholine (2.15 mmol) and 0.28 ml isobutyl chloroformate (2.15 mmol) at -15°C under nitrogen. After 15 min of stirring, a solution of 0.9 phenylenediamine mustard (3.33 mmol) and 0.37 ml N-methylmorpholine (3.33 mmol) in a mixture of 7 ml of DMF and 3 ml of de-aerated water was added. The reaction mixture was then stirred at that temperature for an additional 2 h and at room temperature overnight. The solvent was evaporated under reduced pressure. The residue was dissolved in 100 ml ethyl acetate, washed with saturated sodium bicarbonate, water and cold I M HCl and dried over $MgSO_4$. Upon removal of the solvent, a crude brown product was obtained. The derivatives were purified from ethylacetate/dichloromethane (1/1) through silica gel column chromatography. The products were characterized by TLC: gly . EtOAC : Rf - 0.72; Gly-Leu-Phe : eluents $EtOAc/CH_2Cl_2$ 1/1 : $R_f = 0.49$, Gly-Phe-Ala-Leu : eluents $EtOAc/CH_2Cl_2/MeOH$ 1/1/0/02 : $R_f = 0.35$ or by ^1H-NMR spectroscopy in $CDCl_3$ - Gly - $\delta = 7.37$ ppm and $\delta = 6.63$ ppm, $J = 9.01$ Hz : aromatic protons PDM, $\delta = 3.99$ ppm CH_2 Gly, $\delta = 3.91$ ppm and $\delta = 3.60$ ppm. CH_2 chloroethyl PDM, $\delta = 1.44$ ppm : CH_3 Boc. Gly-Leu-Phe : $\delta = 7.23$ ppm : aromatic protons : Phe,

$\delta = 4.62$ ppm : H_x Phe, $\delta = 4.43$ ppm : α-CH Leu, $\delta = 3.67$ ppm : CH_2 Gly, $\delta = 3.00$ ppm and $\delta = 3.19$ ppm : H_a and H_b Phe, $\delta = 1.62$ ppm : CH Leu, $\delta = 1.51$ ppm : CH_2 Leu, $\delta = 1.44$ ppm, CH_3 Boc. Gly-Phe-Ala-Leu : $\delta = 7.26$ ppm: aromatic protons Phe, $\delta = 4.66$ ppm : H_x Phe, $\delta = 4.44$ppm : α-CH Leu, $\delta = 4.34$ ppm : α-CH Ala, $\delta = 3.52$ ppm : CH_2 Gly, $\delta = 3.05$ ppm and $\delta = 2.72$ ppm : H_a and H_b Phe, $\delta = 1.70$ - 1.45 ppm CH_2 and CH Leu, $\delta = 1.23$ ppm : CH_3 Ala, $\delta = 1.44$ ppm : CH_3 Boc.

c) Removal of the Boc protecting group

A solution of Boc-spacer-PDM (0.304 mmol) in 1.5 ml of 50% trifluoroacetic acid/methylene chloride reagent was stirred at room temperature for 30 min. The excess reagent was removed by evaporation under reduced pressure, the residue was triturated several times with dry ether, and the solid product was dried in vacuo over P_2O_5 and NaOH pellets. The product was characterised by TLC (H_2O/EtOAc/HOAc/nBuOH 1/1/1/1: Gly : R_f = 0.6, Gly-Leu-Phe : R_f = 0.67, Gly-Phe-Ala-Leu : R_f = 0.61) and by ^{1}H-NMR spectroscopy in $CDCl_3$: no more signals of the Boc group could be detected.

4-nitrophenyl chloroformate activation of PHEG

0.25g of PHEG (1.45 mmol units) and 16 mg 4-dimethylaminopyridine (0.13 mmol) were dissolved in 0.25 ml of a NMP/pyridine solution (4/1 v/v), 0.176g of chloroformate (0.87 mmol) was added at 0°C. After 4 h reaction at 0°C, the reaction mixture was precipitated in a mixture of anhydrous ether/ethanol (2/1 v/v). A white precipitate was collected and washed repeatedly with the same mixture. The product was finally dried. The carbonate content was determined by UV analysis after alkaline hydrolysis in NaOH ($\lambda_M = 4.02$ nm, $E_M = 18400$ mol^{-1} em^{-1}).

Preparation of polymeric-PDM-conjugates

0.1g of activated polymer (degree of activation : 4 mol %) and 9.66 mg of Gly-Leu-Phe-PDM (0.015 mmol) were dissolved in a 7.5 ml NMP/pyridine solution (4/1 v/v). After 48 h, the conjugate was separated by precipitation in an anhydrous ether/ethanol mixture (2/1 v/v). The product was washed and dried. The degree of PDM substitution in the conjugates was determined by chloride ion selective electrode after alkaline hydrolysis in NaOH, by UV spectrometry ($\lambda_M = 272$ nm $e_M = 17400$ $mol^{-1}cm^{-1}$) and by ^{1}H-NMR spectroscopy in DMSO-d^6.

Hydrolytic stability of PDM and Gly-PDM

The hydrolytic stability was investigated at 20°C in phosphate buffer pH = 7.4 (0.001 M KH_2PO_4) and pH = 5.2 (0.01M Na_2HPO_4/citric acid). 3 mg of PDM or Gly-PDM were dissolved in 40 ml of buffer and at regular times samples were withdrawn and analyzed using RP-HPLC (column: Zorbax ODS C18 4.6mm x 150 mm flow : 1ml/min injection volume : 20 μl). When evaluating PDM in buffer of physiological pH, 0.51 mg $Na_2S_2O_4$ were simultaneously added to the incubation mixture to prevent oxidation of the mustard. For analysis of PDM, the mobile phase consisted of acetonitrile/acetate buffer (pH = 4, 0.05 M) 40/60 and the products were detected by UV at 258 nm. For Gly-PDM, the mobile phase was methanol/phosphate buffer (0.01M pH = 7.8) 60/40. UV detection was carried out at 272 nm. The percentage of hydrolysis of the mustard was calculated by comparing the area under the curve (AUC) at a certain time to the AUC at the start of the reaction. The retention times of PDM and Gly-PDM are 7.2 min, respectively 4.5 min.

Hydrolytic Stability of Polymeric-peptidyl-PDM Conjugates

The hydrolysis of various polymeric PDM derivatives listed in Tables 1 and 2 below was investigated at 20 °C in phosphate buffer at pH = 7.4 (0.1 M KH_2PO_4) and at pH = 5.2 (0.01 M Na_2HPO_4/citric acid) by a chloride ion selective elctrode. 10 mg of polymer were dissolved in 10 ml of buffer. The concentration of free chloride ions in solution was directly measuered after calibration with standard solutions of known chloride content.

Dynamic laser light scattering

Solutions containing variable concentrations of the PHEG-peptidyl-DM conjugates in duct-free water were filtered and immediately analuzed at 25 °C using a DLS-700 dynamic light scattering spectrophotometer from Polymer Laboratories.

Enzymatic Stability of Polymeric-peptidyl-PDM Conjugates

Degradation in the presence of Collagenase Type IV

Collagenase IV from *Clostridium histolytcum* (Sigma Chem. Co.) was dissolved in phosphate buffered saline (PBS) to poduce concentrations of 4.5 mg/ml, 1 mg/ml and 0.1 mg/ml. 1 mg PHEG-peptidylPDM conjugate was dissolved in 0.6 ml PBS, 0.4 ml of enzyme solution were added and the mixture was incubated at 37 °C. At appropriate times, 0.15 ml samples were withdrawn and analysed by RP-HPLC (column: Zorbax ODS C18 4.6 mm x 150 mm, flow : 1ml/min, injection volume : 20 μl, UV detection at 265 nm) using a mixture of acetonitrile/acetic acid 60/40 pH-4, 0.05 M). Characterisation and calibration of released products was carried out using PDM, Phe-PDM or Leu-PDM solutions of known concentration. The released di- or tri-peptides were analyzed using a

Beckmann Dabs Amino Acid Kit after collection and freeze-drying of the eluting peptidyl-PDM derivatives.

Degradation in the presence of Cathepsin B

The release of PDM in the presence of cathepsin B from bovine spleen was studied at 37 °C in buffer pH = 5.2. 1 mg of conjugate was dissolved in 750 μl phosphate buffer (0.2 M Na_2HPO_4/citric acid), 100 μl of a 50 mM reduced glutathione solution and finally 50 μl of the cathepsin B stock solution (2.5 mg/ml) were added. Samples were taken at appropriate times and analyzed by HPLC as described above.

Degradation in the presence of Tritosomes

The rate of PDM release of the conjugates during incubation with preparations of isolated rat liver lysosomal enzymes (tritosomes) was studied at 37 °C in buffer pH = 5.2. 1 mg of conjugate was dissolved in 400 μl phosphate buffer (0.2 M Na_2HPO_4/citric acid, 0.2 % Triton X-100 w/v), 100 μl of a 10 mM EDTA solution, 100 μl of a 50 mM reduced glutathione solution and finally 400 μl of the tritosomes were added. Samples were taken at appropriate times and analyzed by HPLC as described above.

The results obtained in the above studies are listed in Table 1 to 3 below.

Table 1
(Molar substitution of PDM in % for the PHEG-peptidyl-PDM conjugates determined by ^{1}H-NMR, UV-spectroscopy and elemental analysis)

Peptidyl moiety	^{1}H-NMR	UV	Elem. Anal.
Gly	2.4	2.3	2.6
Gly-Leu-Phe	2.1	1.7	2.0
Gly-Phe-Ala-Leu	2.4	2.5	2.5

Table 2
(Hydrodynamic diameter in nm for the PHEG-peptidyl-PDM conjugates as determined by the dynamic laser light scattering experiments.

Polymer conjugate	Deg. of subst. (mole%)	Concn. (mg/ml)	Diameter (nm)
PHEG	-	10.0	7.5
PHEG-Gly-PDM	2.3	11.0	17.0 and 137.0
		1.4	20.6 and 127.6
PHEG-Gly-Leu-Phe-PDM	1.7	10.7	24.9 and 147.2
		1.56	29.3 and 184.3
PHEG-Gly-Phe Ala-Leu-PDM	2.5	1.5	18.7 and 139.2
PHEG-alaninamide	1.5	5.28	8.3

Table 3
Calculated half-lives (in minutes) for PDM and Gly-PDM in buffers of lsyosomal and physiological pH at 20 °C

Buffer (pH)	PDM	Gly-PDM
5.5	75	240
7.4	11	240

The results obtained in the above studies are also depicted in Figs. 1 to 3 which clearly demonstrate an improved stability for the polymeric-peptidyl derivative compared to the free-form nitrogen mustard, which is

likely to be due to electron withdrawal and chemical stabilisation or the formation of aggregates. The influence of an aminoacid sequence of the spacer on the enzymatic stability of the macromolecular drug conjugate was investigated under different conditions. Accordingly, the conjugate was incubated in buffers of lysosomal and physiological pH and in presence of lysosomal and tumour-associated enzymes. In vitro cytoxicity studies using different cell lines were carried out, which indicated a considerable decrease in cell toxicity for the polymeric PDM conjugates.

Test Example 1

Cytotoxicity against MCF7 wt (human breast carcinoma) cells (wild type)

(1) Preparation of cell culture

MCF7 wt cells were suspended in a solution of RPMI-medium (5% Fetal Calf Serum and 1% L-glutamine) after removal rom their flasks with trypsin. The solution contained 10^4 cell/ml. The cells were added to flat-bottomed 96-welled plates and incubated for 24 hours.

(2) Addition of nitrogen mustards

(i) Addition of free PDM

2mg PDM were dissolved in 2 ml of distilled water and the solution was filtered. A solution of 2 mg/ml of Collagenase Type IV (ex Sigma) was made up and filtered. 100 μM stock solutions of PDM in RPMI-medium, with and without 10 μg/ml Collagenase Type IV, were made up and further diluted to 50, 10, 1 and 0.1 μM. After removal of the medium,

200 μl of each solution was added to the wells, and the plates were incubated for 72 hours.

(ii) Addition of PHEG-Gly-Phe-Ala-Leu-PDM (hereinafter "P-O-PDM")
Stock solutions of 100 μM (based on PDM) of P-O-PDM were made up in RPMI-medium, with and without 10 μg/ml Collagenase Type IV, filtered and further diluted as for free PDM above. After removal of the medium, 200 μl of each solution was added to the wells, and the plates were incubated for 72 hours.

(3) MTT Test

20 μl of a 5 mg/ml MTT solution in PBS was added to each well, and the cells were incubated for 4 hours. After removal of the medium, 200 μl of DMSO was added to each well and the cells were incubated for 30 mins. The absorbance at 550 nm was read in a microtitre plate reader (EL 311 Microplate Autoreader Biotek Instruments). The results are shown in Fig. 5 and in Table 4 below.

Test Example 2
Cytotoxicity against C26 (murine colorectal carcinoma) cells

Test example 1 was repeated with the exception that C26 cells were used instead of MCF7 wt cells. The results obtained are shown in Fig. 6 and in Table 4 below.

Test Example 3
Cytotoxicity against A-375 M (human melanoma cells - metastatic variant)

(1) Cell preparation

(i) Fresh, as-supplied cell medium (FCM) containing A-374 M cells was used.

(ii) Conditioned cell medium (CCM) was prepared by collecting medium containing tumour-secreted proteases from A-375M cells (containing approximately 750 x 10^4 cells in 10ml of DME-medium) after incubation of these cells in their flasks for 72 hours. This medium was used as such, without further dilution or purification.

(2) Addition of nitrogen mustards

(i) Addition of free PDM
4mg PDM were dissolved in 2 ml of distilled water and the solution was filtered. A 100 μM stock solution of PDM in DME-medium was made up and further diluted to 50, 10, 1 and 0.1 μM. After removal of the medium, 100 μl of each solution together with 100 μl of FCM or CCM were added to the wells, and the plates were incubated for 72 hours.

(ii) Addition of PHEG-Gly-Phe-Ala-Leu-PDM (P-O-PDM)
Stock solutions of 100 μM (based on PDM) of P-O-PDM were made up in DME-medium, filtered and further diluted as for free PDM above. After removal of the medium, 100 μl of each solution together with 100 μl of FCM or CCM were added to the wells, and the plates were incubated for 72 hours.

(3) MTT Test

The MTT test procedure as described in Test Example 1 was performed. The results obtained are shown in Fig. 7 and in Table 4 below.

Table 4 - (IC_{50} values for various cell lines after 72 hours)

Test Material	MCF7 wt	C26	A-375M
PDM + FCM	13 μM	2.8 μM	2.5 μM
P-O-PDM+FCM	>100 μM	>100 μM	>100 μM
PDM+CCM	9 μM	2.3 μM	2.5 μM
P-O-PDM+CCM	80 μM	24 μM	100 μM[(1)]

[(1)] - IC_{40}

Test Example 4 (In vivo test in mice)

Female BALB/c mice (5-6 weeks old) were subcutaneously inoculated with 10^5 C26 murine colorectal carcinoma cells. When the tumours were palpable, groups of 5 animlas were treated *in vivo* on each of three consecutive days with either free PDM (2 or 5 mg/kg), P-O-PDM (2, 5 or 8 mg/kg related to the PDM component) or saline (as a control). The animals were weighed daily and tumour growth measured. Animals receiving free PDM (5 mg/kg) showed rapid weight loss and were put down after 4-6 days following treatment due to treatment-related toxicity. Animals treated with PDM (2 mg/kg) showed tumour growth identical with saline-treated control animals and they were put down after 10-12 days due to tumour burden. Animals treated with P-O-PDM fared better; there was no measurable toxicity and tumour growth was delayed at higher doses, although tumours did eventually grow and animals were eventually put down due to tumour burden. However, one animal was effectively cured and remains alive and tumour-free after 46 days.

The results obtained are shown in attached Figs. 8 to 12. Survival times (days) were as follows:-

Control:	8, 9, 11, 11, 11
PDM (2 mg/kg):	11, 11, 11, 11, 11
PDM (5 mg/kg):	5, 6, 6, 6, 7
P-O-PDM (2mg/kg):	9, 11, 11, 11, 11
P-O-PDM (5mg/kg):	11, 11, 18, 18, >46
P-O-PDM (8mg/kg):	17, 17, 18, 18, 18.

CLAIMS

1. An anti-tumour agent comprising a macromolecular carrier moiety; an active alkylating moiety which is a nitrogen mustard; and a stabilising moiety linking the active alkylating moiety with the carrier moiety, said stabilising moiety being an oligopeptide which is capable of being cleaved by a tumour-associated protease and which stabilises the active alkylating moiety by electron withdrawal and/or by inducing formation of aggregates.

2. An agent as claimed in claim 1, wherein the oligopeptide is one which can be cleaved by a metalloproteinase or a lysosomal enzyme.

3. An agent as claimed in claim 1 or 2, wherein the oligopeptide is a tetra-, penta- or hexa-peptide.

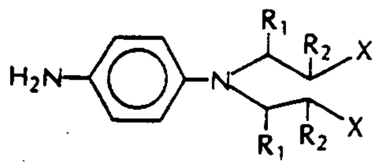
4. An agent as claimed in claim 1, 2 or 3, wherein the oligopeptide is a tetrapeptide.

5. An agent as claimed in claim 4, wherein the tetrapeptide is (Leu or Gly)-(Ala or Leu)-(Phe or Leu)-(Gly or Ala).

6. An agent as claimed in claim 1 or 2, wherein the oligopeptide terminates with, or consists of, the tetra-peptide, Gly-Phe-Ala-Leu, Gly-Phe-Leu-Gly or Ala-Leu-Ala-Leu.

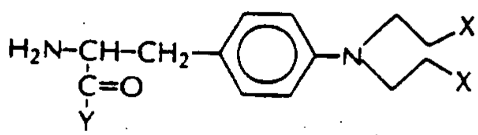
7. An agent as claimed in any preceding claim, wherein the active alkylating moiety is N,N-di(2-chloroethyl)-4-phenylenediamine,

$CH_3N(CH_2CH_2Cl)_2$,



where R_1 and R_2 are independently selected fro H, CH_3 or allyl and X is Cl, Br, I, mesyl or tosyla

or



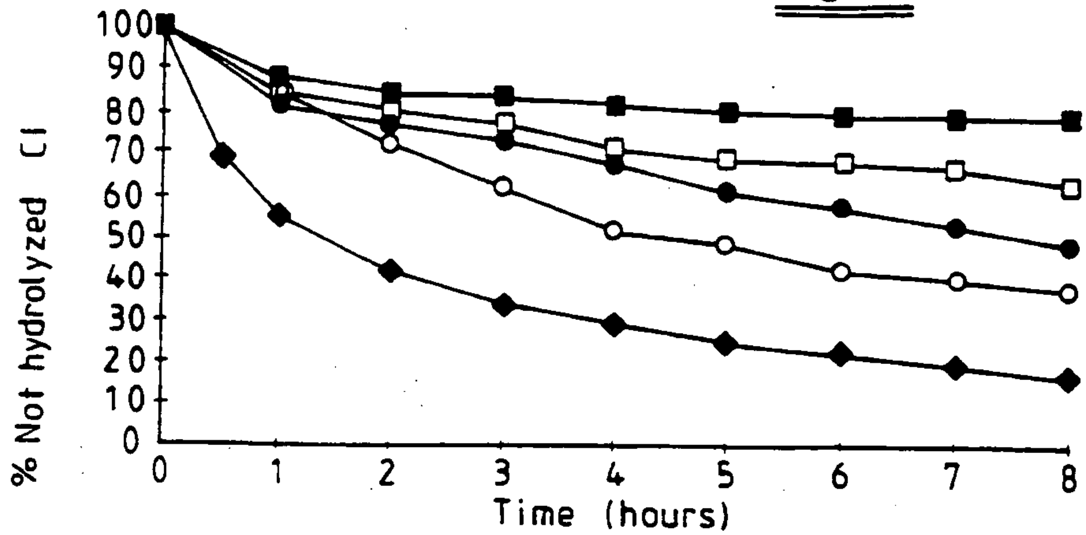
where Y is OH, alkoxy or NH_2 and X is as defined above

8. An agent as claimed in any one of claims 1 to 6, wherein the active alkylating moiety is N,N-di(2-chloroethyl)-4-phenylenediamine.

9. A method of treating a tumour in a patient comprising the step of administering to the patient an anti-tumour agent as claimed in any preceding claim.

Stability at pH=5·2

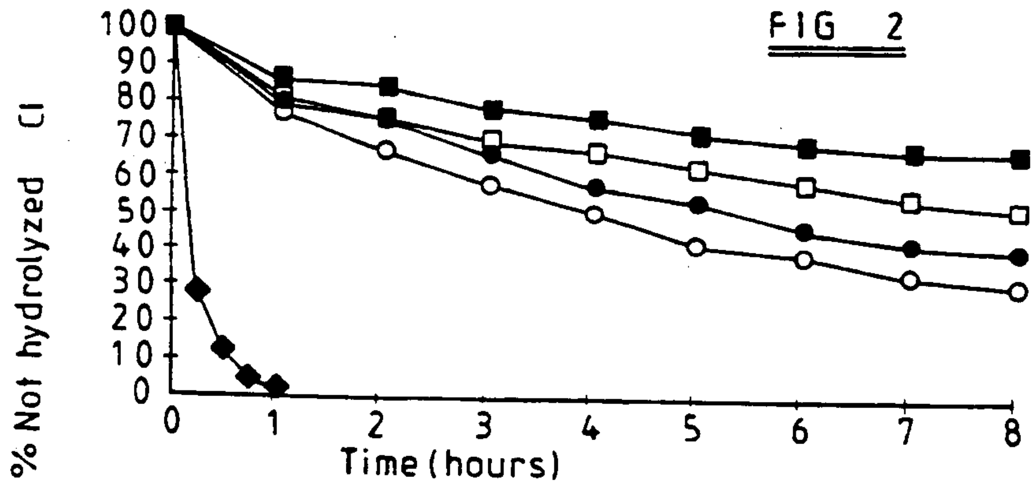
FIG 1



Time course of hydrolysis of PDM and its derivatives in buffer of lysosomal pH (◆):PDM, (○):gly-PDM, (●):PHEG-gly-PDM, (□):PHEG-gly-phe-ala-leu-PDM, (■):PHEG-gly-leu-phe-PDM

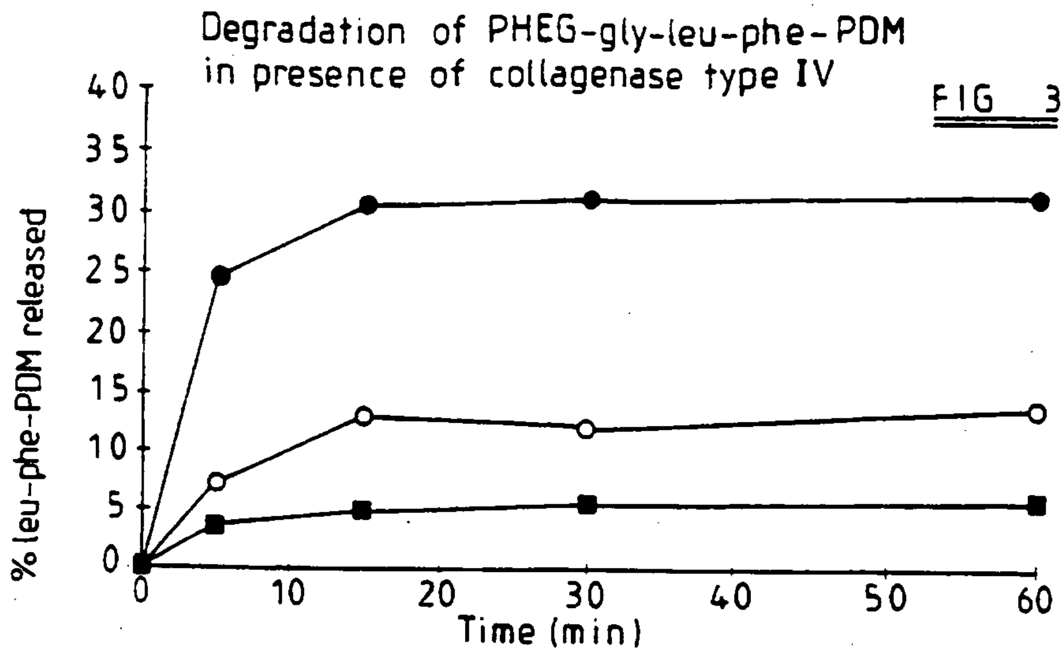
Stability at pH=7·4

FIG 2



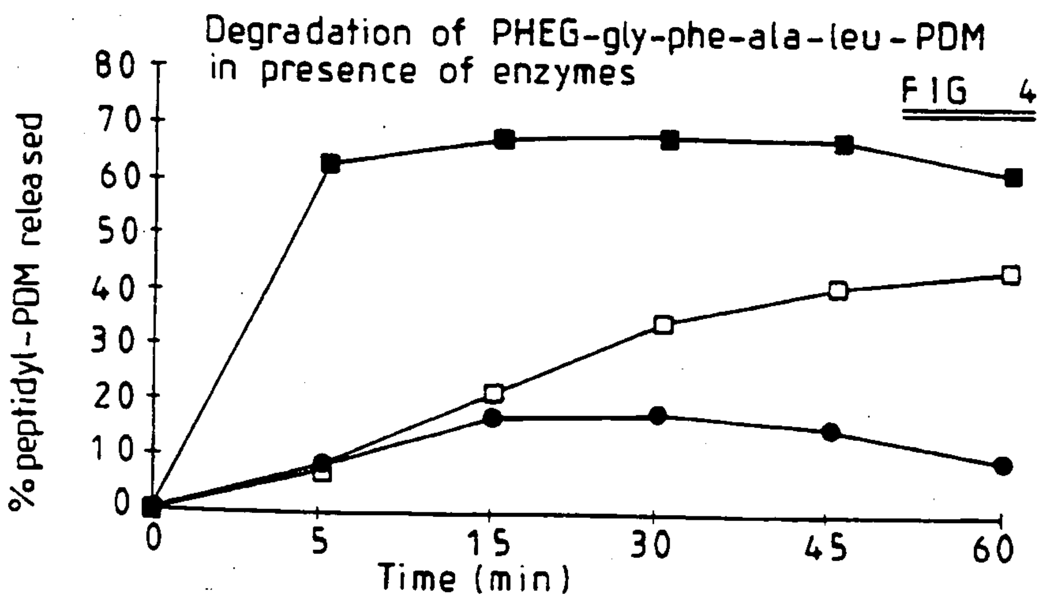
Time course of hydrolysis of PDM and its derivatives in buffer of physiological pH (◆):PDM, (○): gly-PDM, (●)PHEG-gly-PDM, (□):PHEG-gly-phe-ala-leu-PDM, (■): PHEG-gly-leu-phe-PDM

FIG 3



Time course of enzymatic hydrolysis of PHEG-gly-leu-phe-PDM with different concentrations of collagenase type IV (■): 0·1mg/ml, (○): 1mg/ml, (●): 4·5mg/ml

FIG 4



Time course of hydrolysis of PHEG-gly-phe-ala-leu-PDM in presence of different enzymes (●): tritosomes, (□): cathepsin B, (■): collagenase type IV mg/ml

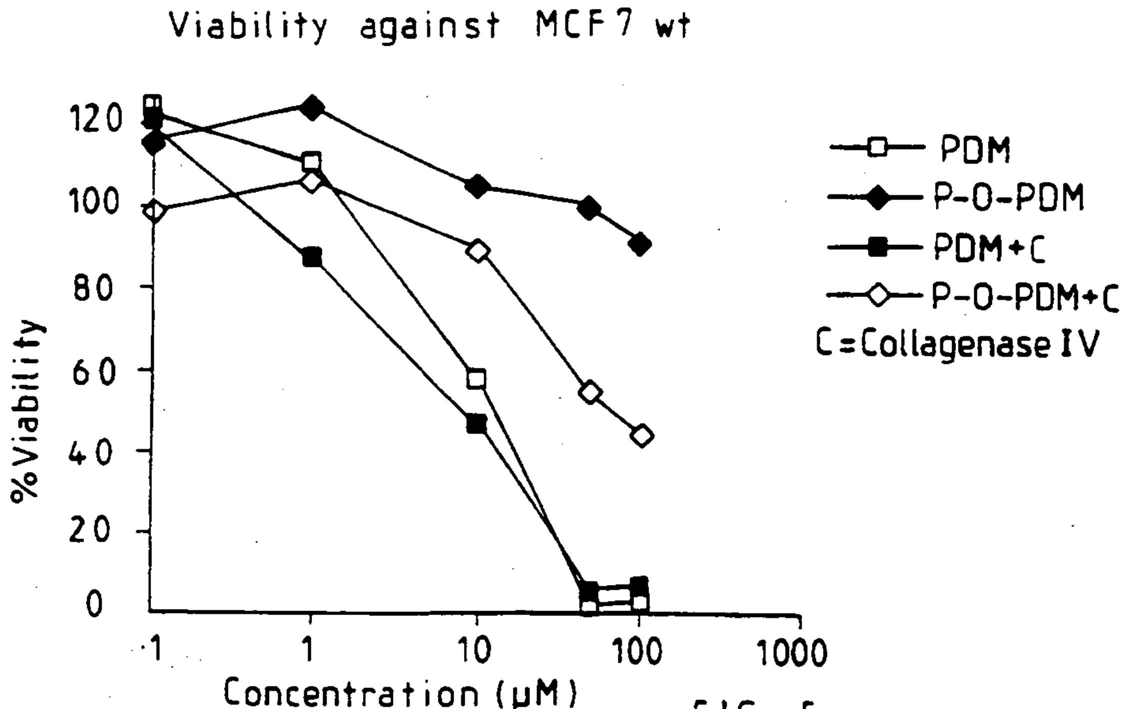

Viability against MCF7 wt
120
100
80
60
40
20
0
% Viability
·1
1
10
100
1000
Concentration (μM)
PDM
P-O-PDM
PDM+C
P-O-PDM+C
C=Collagenase IV

FIG 5

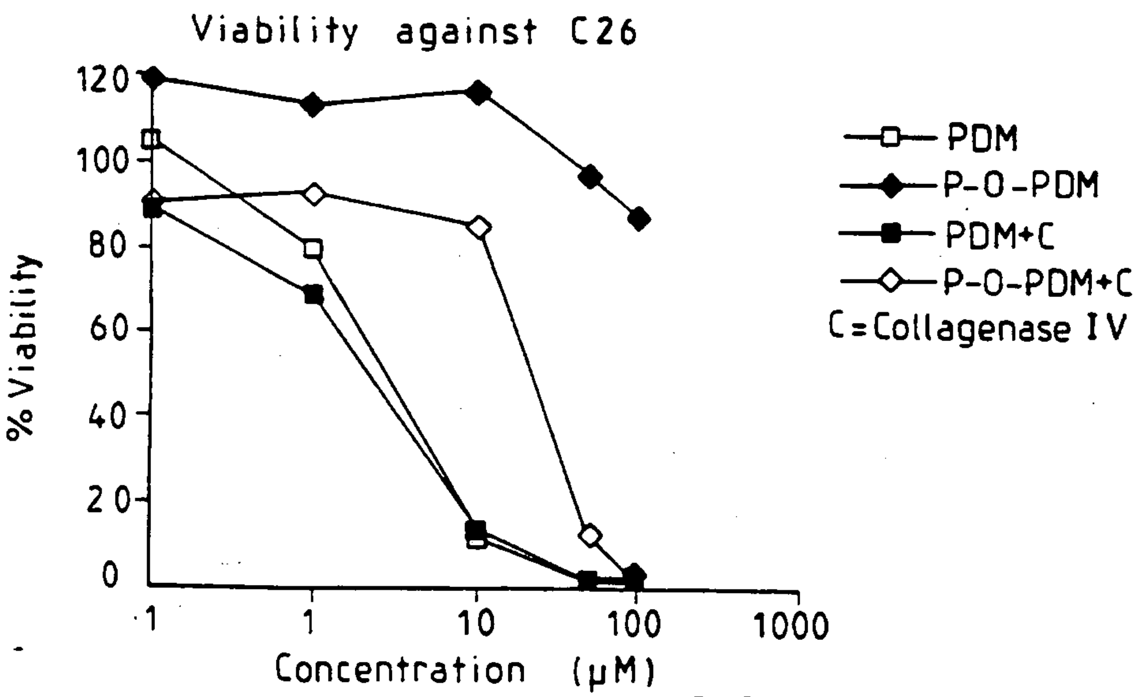

Viability against C26
120
100
80
60
40
20
0
% Viability
·1
1
10
100
1000
Concentration (μM)
PDM
P-O-PDM
PDM+C
P-O-PDM+C
C=Collagenase IV

FIG 6

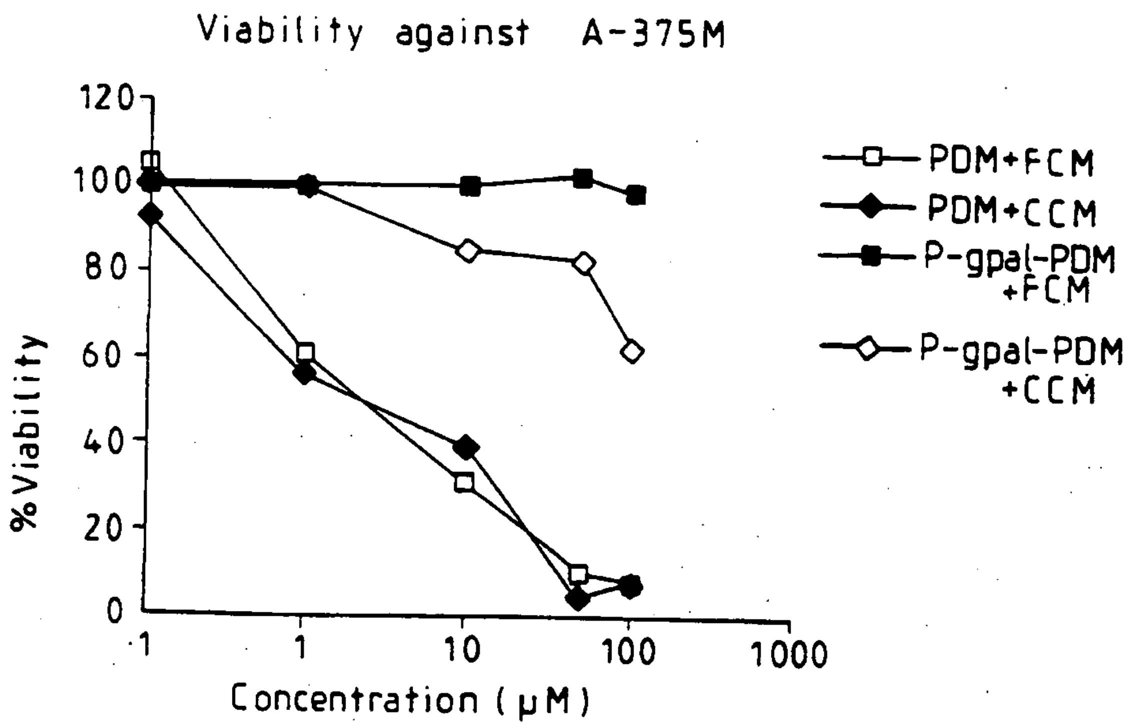

Viability against A-375M
120
100
80
60
40
20
0
% Viability
·1
1
10
100
1000
Concentration (μM)
PDM+FCM
PDM+CCM
P-gpal-PDM +FCM
P-gpal-PDM +CCM

FIG 7

Influence of free PDM on sc. C26 growth

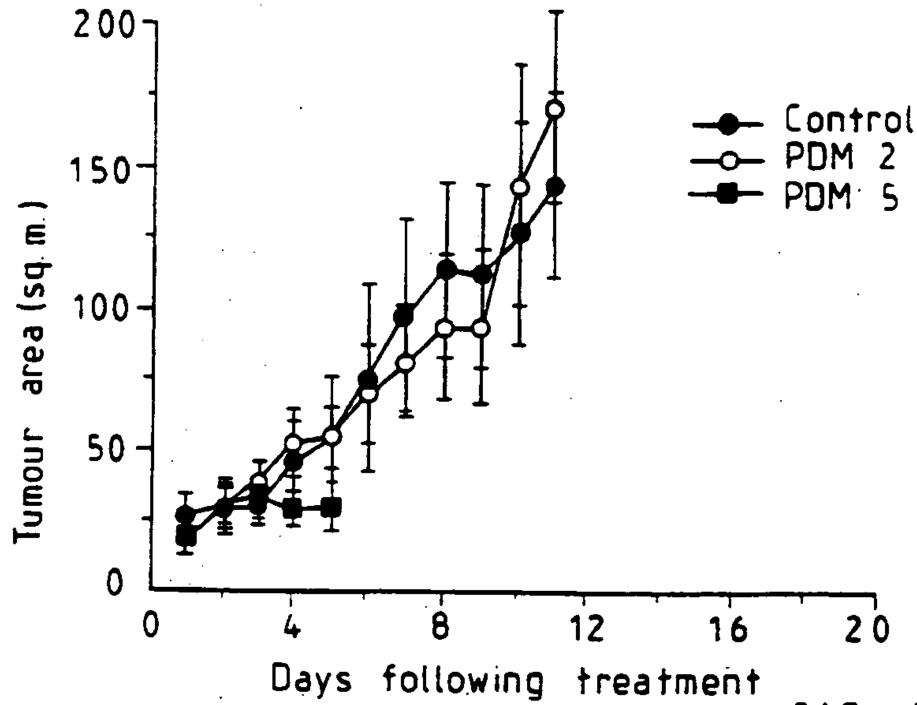


FIG 8

Influence of P-O-PDM on sc. C26 growth

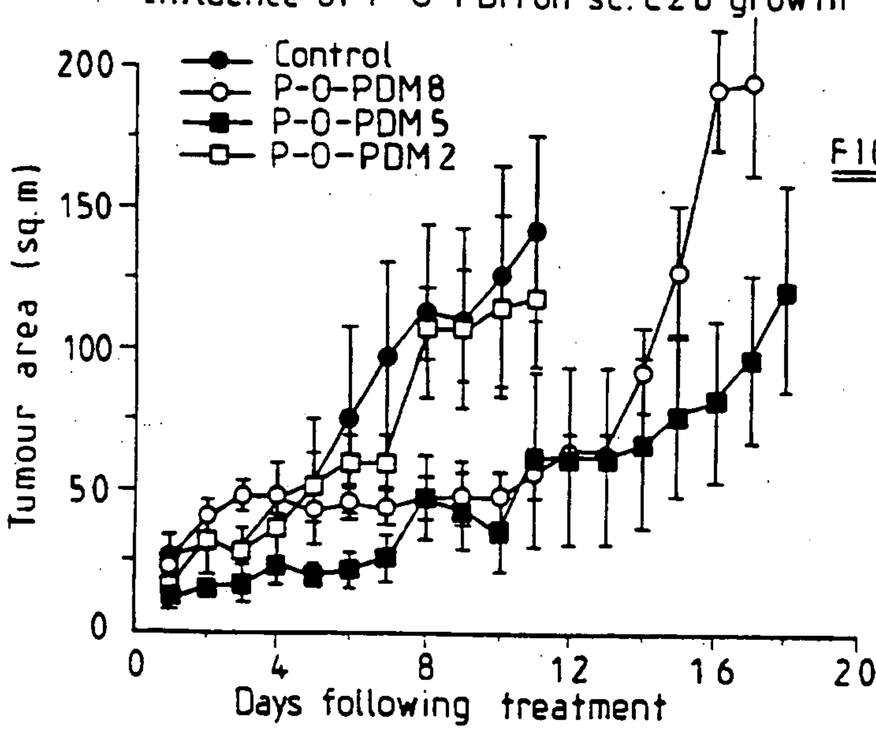


FIG 9

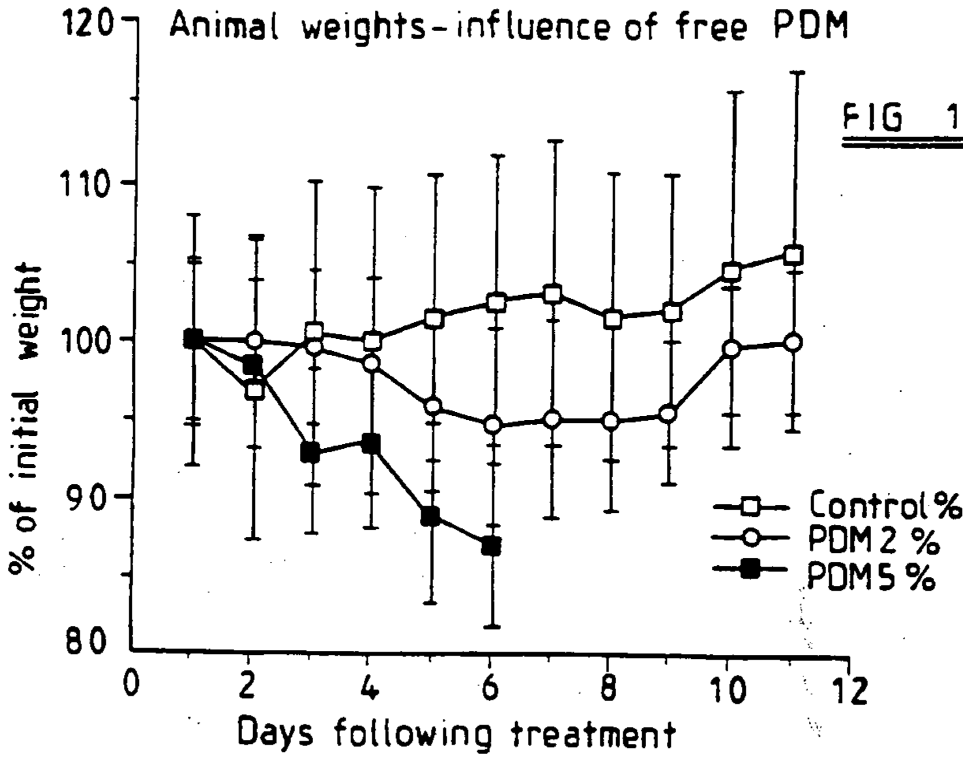

Animal weights-influence of free PDM
120
110
100
90
80
% of initial weight
0
2
4
6
8
10
12
Days following treatment
Control %
PDM 2 %
PDM 5 %

FIG 10

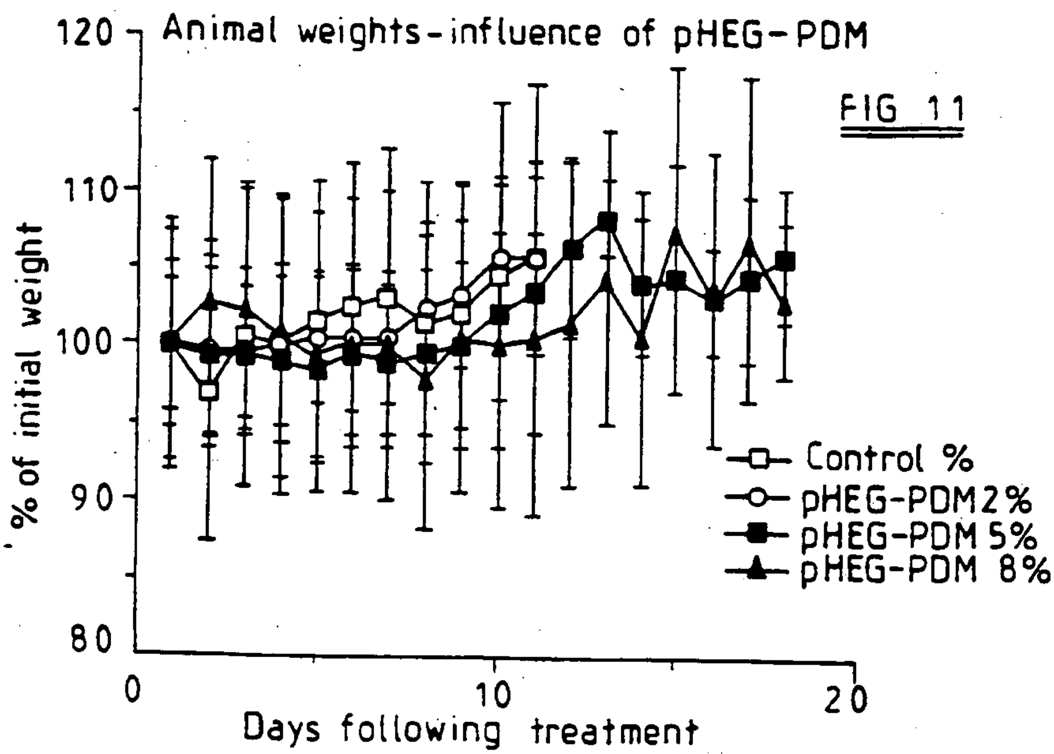

Animal weights-influence of pHEG-PDM
120
110
100
90
80
% of initial weight
0
10
20
Days following treatment
Control %
pHEG-PDM 2%
pHEG-PDM 5%
pHEG-PDM 8%

FIG 11

Survival graph: Mice with s.c.26 treated with pHEG-PDM

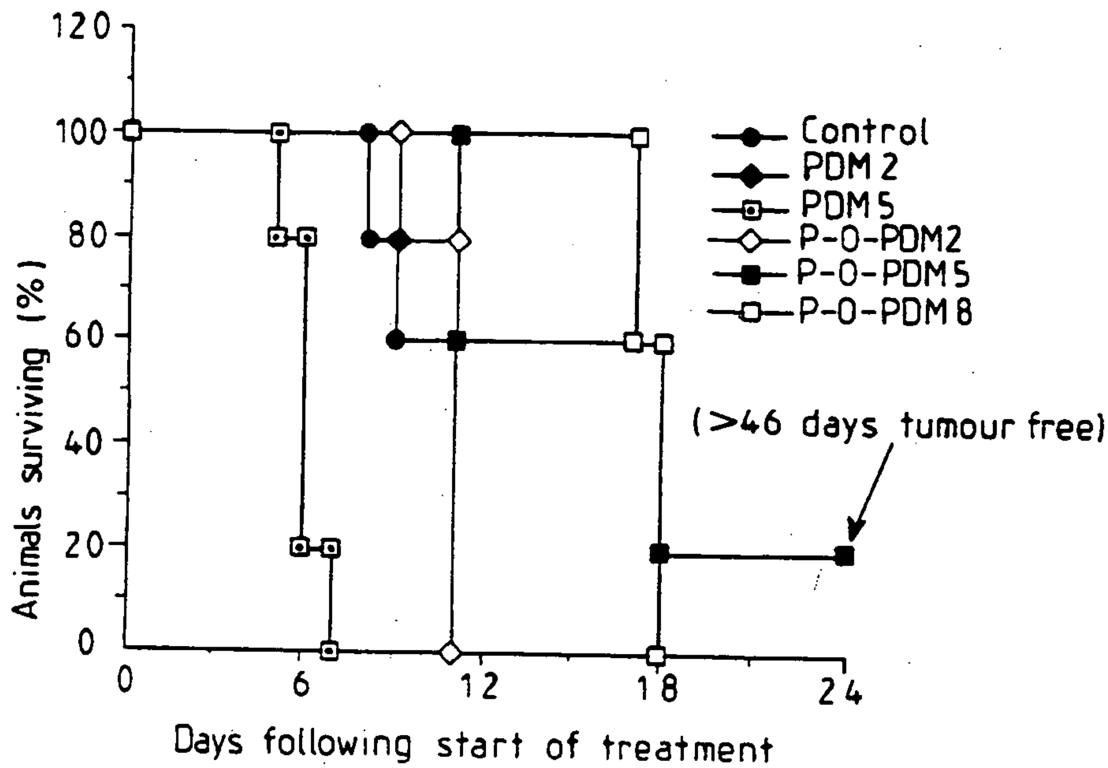


FIG 12